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Processing, quantification of polyphenols and anti-oxidant assay of cocoa beans

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ABSTRACT

Polyphenols widely distributed in plants, fruits and vegetables have received considerable attention because of their physiological functions including antioxidant, antimutagenic and/or cancer preventive activities. The present study aims to process cocoa beans and to estimate the quantity of epicatechin after processing. The seeds of *Theobroma cocoa* were subjected to two different treatments. One set of seeds were allowed to boil and other for fermentation. Both the boiled seeds and fermented seeds were further grouped into two. The first group of boiled seeds was dried and deshelled (BNR), second group of seeds were dried and roasted (BR). The fermented seeds were then dried under sunlight and deshelled without roasting (FNR) and the other group of seeds was roasted and deshelled (FR). The obtained seeds were ground to cocoa liquor and polyphenols were extracted using various solvents and concentrated by rotary evaporation, and these solutions were subjected to HPLC analysis and eluted compounds were detected by monitoring UV absorbance at 280 nm. The calibration curves were compared against the standard which was run at same nm. The obtained calibration curves identified the presence of the polyphenolic compound-epicatechin at 6 min. The sample BNR possessed the maximum concentration of o.938mg/g of epicatechin whereas the sample FR doesn't obtained any peak representing the presence of epicatechin. Hence the study concludes that processing that involves fermentation and heat reduced epicatechin content as well as antioxidant activity.

Introduction

Polyphenols widely distributed in plants, fruits, and vegetables have received considerable attention because of their physiological functions including antioxidant, antimutagenic and/or cancer preventive activities (Saliva *et al.*, 1992; Bomser *et al.*, 1999; and Zhao *et al.*, 1999). The initial processing steps that cocoa beans must undergo at the site of cultivation are fermentation, drying and deshelling.

Beans are sterilized, roasted and ground to become cocoa liquor and then this can be made into cocoa powder or used directly in chocolate product (Gu, Lw *et al.*, 2006).Fermentation is also considered to be one of the major steps that affect polyphenol content, between day 2 and day 3, epicatechin content has been shown to decrease sharply, which could indicate that it is either taken up in the formation of larger tannins or lost in the fluids that drain away during fermentation (Kim and Keeny,1984).

The present study aims to process cocoa beans and estimate the quantity of epicatechin after processing.

Methodology

Selection of Cocoa seeds:

Fresh cocoa fruits of *Theobroma Cocoa* were obtained from TamilNadu Agricultural Farm, Karumandurai, TamilNadu, India.

Cocoa seeds from ripe pods were hand dissected using a scalp and freed from stem and foamy pulp or water pulp.

The seed coat was cut away leaving the cotyledons, hypocotyls axis and root radical.

Processing of Cocoa seeds:

The seeds of *Theobroma cocoa* were subjected to two different treatments. One set of seeds was allowed to boil at an internal bean temperature of 95^0C for five minutes. The boiled seeds were further grouped into two. The first group of seeds was dried under sunlight for two days until the moisture get reduced to 4% and deshelled (BNR) second group of seeds were dried and roasted (BR). The other set of seeds were allowed for natural aerobic fermentation for five days with occasional stirring. The processed seeds were then dried under sunlight for two days until the moisture get reduced to 4%. After complete fermentation and drying, the seeds were grouped into two same as to boiled seeds. The one group of seeds were deshelled without roasting (FNR) and the other group of seeds were roasted (FR) and deshelled.

Sample preparation for epicatechin from cocoa liquor:

After various treatments the obtained seeds were subjected for its polyphenol-epicatechin content. The extraction of samples was done according to the method used by Natsume *et al.*, (2000). The extraction procedure briefly involves trituration of 10g of cocoa liquor 3 times with a 5-fold volume of *n*-hexane (3 x 50 ml) at room temperature for 30 min in order to remove most of the fats. The defatted cocoa liquor (0.25 g) was extracted with 80% (v/v) acetone (3 x 25 ml) at 80 ºC, then the mixture was filtered through a filter paper (Whatman No. 1) using a buchner funnel. This resulting solution was considered as polyphenol solution. The obtained 30ml of each polyphenol

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solution from various processed cocoa liquor was concentrated to 3ml before HPLC analysis.

HPLC analysis:

The HPLC apparatus used was a shimadzu instrument (UV detector) and an eluted compound was detected by monitoring the UV absorbance. Epicatechin standard of 90% purity was obtained from SIGMA ALDRICH (Flucka) co, USA. The HPLC grade analytical solvents used for extraction were acetone and hexane and for running the samples in HPLC acetonitrile, trifluoro acetic acid and water were used.

Analysis of Epicatechin:

Ten microlitres of each polyphenol solution was analyzed on HPLC with a C18 column (250 mm x 4.6 mm I.D., 5 um), by using the solvents (A):0.1% trifluro acetic acid in acetonitrile (CH_3CN) and (B):0.1% trifluro acetic acid in water. Elution was done with a linear gradient of 0 to 10% A in 5min, 10 to 25% A in 25min and 25 to 100% A in 5min (flow rate 0.8 ml/min).

Quantification of epicatechin in various processed cocoa liquor:

Calibration curves were made from the stock solutions using a quadratic fit for the relationship of area sum versus concentration for the peaks corresponding to each oligomeric class.

DPPH radical scavenging assay:

The scavenging activity was estimated according to the method of Brand-Williams *et al*. (1995). Briefly, methanol extracts of cocoa liquor were diluted to 15, 12.5, 10, 7.5 and 5 mg of defatted cocoa liquor per ml and 50 µl of each dilution was added to 3.992 ml of 100µM methanol DPPH* solution. The decrease in absorbance was determined at 515 nm using UV spectrofotometer, Cary 3, Varian (Palo Alto, USA), at 0 min and every 2 min until the reaction reached the plateau. The remaining DPPH* concentration in the reaction medium was calculated from the calibration curve. For each concentration tested, the reaction kinetics was plotted. From these graphs the percentage of the residual DPPH* was determined, and the values were transferred into another graph showing the percentage of the residual DPPH* as a function of the cocoa liquor concentration in the diluted methanol extract.

Results and Discussion

Identifying Polyphenols In Cocoa Liquor

Epicatechin was identified using the standard peak which run at 280 nm (Natsume *et al*., 2000) and identified in all the variety of treatments of cocoa liquor samples like BNR, BR, FNR, and FR. The obtained calibration curves identified the presence of this polypheonlic compound-epicatechin at 6 min. The calibration curves which identified the compound were given in fig 1, 2, 3 and 4. Perhaps, the sample FR doesn't showed any peak representing the presence of the polyphenolic compound epicatechin.

The total phenols in cocoa gets reduced during fermentation to 30% of the initial value and (-) - epicatechin principal substrate of cocoa polyphenol oxidase, is reduced by 90%, with a proportional increase in catechin content. Polyphenol reduction during drying was attributed to non-enzymatic browning from quinine polymerization (Edy sousa de brito *et al*., 2002)

Quantification of Polyphenolic compound in processed bean samples

The table I picturises the quantity of epicatechin and anti-oxidant activity present in processed cocoa beans. The sample BNR ranked first with higher concentration of epicatechin followed by FNR and BR samples whereas there was absence of epicatechin in FR sample. The results revealed that fermentation as well as roasting influences the polyphenolic compound-epicatechin with respect to epicatechin as the processing advances, the polyphenolic contents deteriorates.

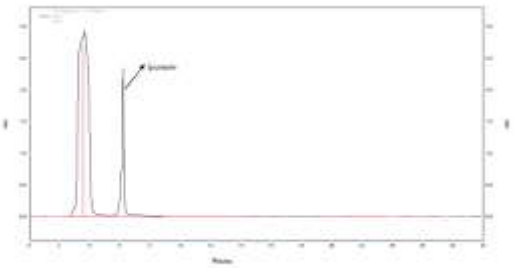

Fig.1 Chromatogram obtained from BNR sample in epicatechin analysis

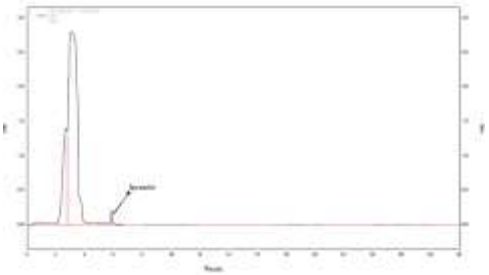

Fig.2 Chromatogram obtained from BR sample in epicatechin analysis

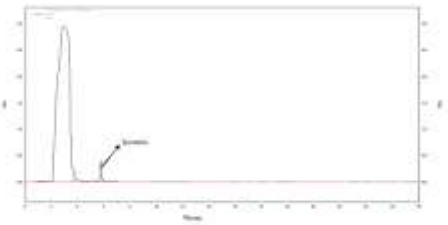

Fig.3Chromatogram obtained from FNR sample in epicatechin analysis

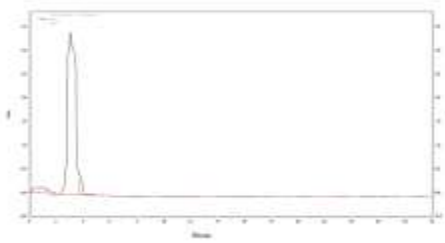

Fig.4 Chromatogram obtained from FR sample in epicatechin analysis

Antioxidant activity of cocoa bean samples:

Antioxidant activity is also dependent on the structure of the free radical-scavenging compounds, substituents present on the rings of the flavanoids and degree of polymerization (Wood, 2002)

From the above table it is evident that antioxidant activity increased where there is minimal processing. The least percent of free radical scavenging observed in FR sample, inferring less quantity of polyphenolic antioxidant compounds. A similar obtained in the study of Stahl, L., *et al*., 2009, stated that hot cocoa drink also retained high levels of antioxidant activity from 92% to 156%. After correction for moisture loss, recoveries in

the chocolate cookies ranged from 88% for total polyphenols to113% for antioxidant activity. Chocolate cakes showed the most dramatic loss in all measures with recoveries of 42% to 54% for ORAC and TP, and 4% to 27% for the flavonol monomers and procyanidins.

EC50 values, the amount of antioxidant necessary to decrease the initial DPPH* radical concentration by 50%, were determined from the graph of scavenging activity plotted against the concentration of DCL. The lowest EC50 indicates the highest ability of the extract to act as DPPH* scavenger. Radical scavenging activities of cocoa liquors were in the following order: the Madagascar > Mexico > Ecuador > Venezuela Sao Tome > Ghana samples. (Redovni kovic, 2009).

Low IC50value indicates higher antioxidant activity. Stem and leaf showed higher free radical scavenging activity (22 and 23 µg/ml) than fruit pulp (59µg/ml) and seed (94µg/ml) (Gahane, *et al*., 2010).

Conclusion

It is concluded from the study that processing influences the polyphenolic compounds. Even though they enhance the aroma of the cocoa beans they are sensitive to extreme heat application and deep fermentation. Fermentation followed by roasting produced deleterious impact on the polyphenolic compounds. Boiling and roasting does not affected the epicatechin content however, it retained optimum amount of epicatechin. On the other hand, fermentation and roasting influenced deeply with regard to free radical scavenging activity which may be due to the concentration and type of polyphenol present in each extract.

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Table I Quantifying epicatechin content in the processed seeds

Sl. No.	Processed Bean Varieties	Quantity of Epicatechin(mg/g)	Antioxidant activity
1	BNR	0.938	79%
2	BR	0.072	76%
3	FNR	0.129	78%
4	FR	-	69%